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EFFECTS OF *ALLIARIA PETIOLATA* (GARLIC MUSTARD) ON *MORCHELLA* (MORELS) *IN VITRO*

A Chapter Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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EFFECTS OF *ALLIARIA PETIOLATA* (GARLIC MUSTARD) LEACHATE ON *MORCHELLA* (MORELS) *IN VITRO*

By Elizabeth Leighton

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science.

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ABSTRACT

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Previous research has shown that *Alliaria petiolata*, garlic mustard, successfully invaded northern North America through allelopathy and suppressing arbuscular and ectomycorrhizal fungi. The research suggests that allelochemicals suppress mycorrhizal fungi's spore viability and infectivity. Morels (*Morchella*) are popular edible fungi that live in habitats threatened by *A. petiolata*, even though their interaction with this plant has not been studied. This study explored whether *Morchella* is suppressed by *A. petiolata* by examining the effects of aqueous root, shoot, and whole plant *A. petiolata* leachates on *Morchella* cultures *in vitro*. Four traits appeared in response to treatment: deformation, line, pigment, and zone of inhibition. The root leachate elicited higher expression of pigment, line, and zone of inhibition traits. *Morchella elata s.l.* cultures were more affected by *A. petiolata* root leachate. *Morchella americana* cultures were likely to express line and pigment traits, but not the zone of inhibition trait. The *in vitro* conditions may not perfectly simulate soil conditions, but the responses in this experiment could occur in nature. Further research is needed to understand whether these traits appear in nature and whether these traits are associated with suppression in nature.

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CHAPTER I

ALLIARIA PETIOLATA ALLELOPATHY AND INVASION IN NORTH AMERICA

Alliaria petiolata Invasion

Garlic mustard, *Alliaria petiolata*, is aggressively invading forest communities throughout much of North America. A great deal of research has been done to understand why it so successfully establishes large stands in North America when it does not do so in its native Europe. Studies have shown that the presence of *A. petiolata* in soil alters both the arbuscular and ectomycorrhizal fungi communities (Callaway *et al.*, 2008; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). However, not all mycorrhizal fungi have been studied; thus, our understanding of *A. petiolata*'s suppression is incomplete. To date, no published studies have reported on whether *A. petiolata* affects any edible mycorrhizal fungi. This study begins to explore whether *A. petiolata* affects the growth of *Morchella*, a highly prized edible mushroom.

Rapid movement across the globe of many organisms has resulted in biological invasions in many environments worldwide. Invasive species are newly established in an environment and dominate in such a way that the environment is damaged (Pimentel *et al.*, 2005). Damage to the environment includes limiting recreational activity, economic productivity, and decreasing biodiversity. Invasive species are estimated to cost the United States $120 billion per year (Pimentel *et al.*,

2005). Because of these costs and damages, many efforts are underway to fully understand the mechanisms of invasion success.

Invasive plants change their invaded habitats both above ground and below ground. Below ground, invasive plants cause changes to the soil environment, including altering chemical properties (Evans *et al.*, 2001) and changing soil microbial communities (Hawkes *et al.*, 2006; Heneghan *et al.*, 2006; Kourtev *et al.*, 2003; Mangla *et al.*, 2008; Rodgers *et al.*, 2008). Studies have established that feedback between soil microbial communities and plant communities drive diversity, success and functioning in both communities (Wardle *et al.*, 2004). For example, an invasive plant can shape the soil microbial community to disadvantage the native plant species (Callaway & Ridenour, 2004).

Allelopathy is one plant mechanism that can alter belowground communities. Negative allelopathy is a mechanism where one organism produces a secondary metabolite—a chemical not necessary for growth or reproduction, but for defense or attack—that inhibits the growth and success of surrounding organisms (Stamp, 2003). Allelopathic organisms can be found in many phylogenetic lineage, and affected organisms can belong to almost any taxon as well. Allelopathy plays a role in plant invasions when the introduced plant produces allelopathic compounds to which native organisms are naïve and susceptible (Hierro & Callaway, 2003).

In the invasive plant's native environment, the neighboring biota are, on average, more resistant to the allelochemicals, and the plant is usually not a dominant part of the plant community (Callaway & Aschehoug, 2000). However, in a naïve, introduced environment, neighboring organisms are more susceptible to the

allelochemical, and the invasive plant may become dominant. This invasion strategy is commonly known as the Novel Weapons Hypothesis (Callaway & Ridenour, 2004). This mechanism has been documented in very few organisms, but for those organisms, it is important for invasion success.

Figure 1. (Left) Basal rosette and mature *Alliaria petiolata* individuals https://microbewiki.kenyon.edu/index.php/Alliaria_Petiolata_and_Mycorrhiza (Right) A. petiolata flowers http://pick4.pick.uga.edu/mp/

In North America, garlic mustard's (*Alliaria petiolata*, Brassicaceae) success is often attributed to allelopathy. *Alliaria petiolata* is a biennial herb that has spread throughout the United States and Canada, where it prefers disturbed, moist, temperate environments (Anderson *et al.*, 1996; Cavers *et al.*, 1979). It has also invaded high quality temperate forests, first documented in Illinois, where its presence leads to a decrease in biodiversity (Nuzzo, 1999). The first-year plants grow into basal rosettes that overwinter into the second year. The mature plant bolts from the rosette, flowers from late spring to early summer, and dies in mid- to late summer. As with all brassicas, mycorrhizae have never been documented in *A. petiolata*, so it is considered to be non-mycorrhizal (Cavers *et al.*, 1979). Studies have found that not

only is *A. petiolata* non-mycorrhizal, but that it produces anti-fungal compounds that suppress mycorrhizae in the soil surrounding it (Callaway & Ridenour, 2004; Cantor, *et al.*, 2011; Stinson *et al.*, 2006).

Alliaria petiolata's Allelochemistry

Alliaria petiolata allelochemistry was discovered when two unique glucosinolates and isothiocyanates isolated from *A. petiolata* suppressed the germination of wheat (*Triticum aestivum*) and cress (*Lepidium sativum*) (Vaughn & Berhow, 1999). Glucosinolates are flavonoids found in nearly all brassicaceous plants and are converted to isothiocyanates by a myrosinase enzyme (Bones & Rossiter, 1996). There are multiple forms of glucosinolates and isothiocyanates, differentiated by their side chains. *Alliaria petiolata* produces the glucosinolates sinigrin, which has an allyl side chain (Figure 2), and glucotropaeolin, which has a benzyl side chain (Figure 3) (Vaughn & Berhow, 1999). Sinigrin and glucotropaeolin are converted by the myrosinase enzyme to allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC) (Figures 2 and 3, respectively). To date, *A. petiolata* is the only plant in North America known to produce sinigrin/AITC and glucotropaeolin/BITC, but there are other plants native to Asia and Europe that produce these compounds (Tsao *et al.*, 2000). Glucosinolates and isothiocyanates primarily serve as defense against herbivores and pathogens (Agrawal & Kurashige, 2003). Sinigrin and glucotropaeolin serve as allelochemicals; sinigrin and glucotropaeolin decreased root radicle elongation in *T. aestivum* and *L. sativum* (Vaughn & Berhow, 1999). Production of sinigrin and glucotropaeolin varied by tissue type (root vs. shoot) and by season. Root tissues contained the highest amounts of glucotropaeolin (15.3 μg/mg in spring, and

50.2 μg/mg), whereas there were small amounts of sinigrin in roots both in May 1998 and October 1998 (3.5 μg/mg and 2.8 μg/mg, respectively) and the shoots tissues in autumn (2.8 μg/mg).

Alliaria petiolata's allelopathy also extends to fungi. Calmes *et al.* (2015) discovered that AITC, BITC, and phenetyl ITC affected cellular function in a pathogenic fungus *Alternaria brassicicola*. These ITCs affected mitochondrial function and increased the production of reactive oxygen species in *A. brassicicola* (Calmes *et al.*, 2015).

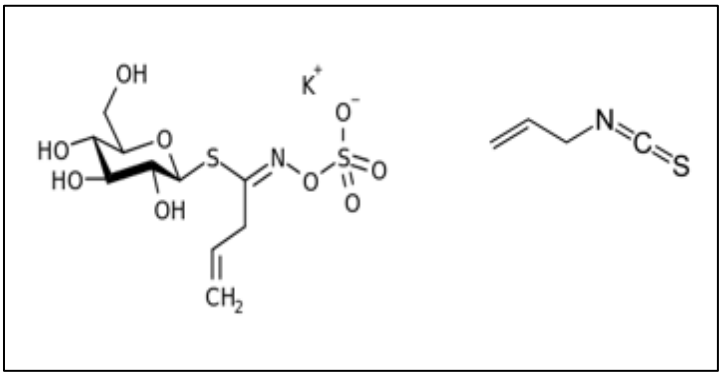

Figure 2. Chemical structures of sinigrin (left) and allyl isthiocyanate (right) as provided, respectively, By IAMGOOMBA - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=48139526 ; Public Domain

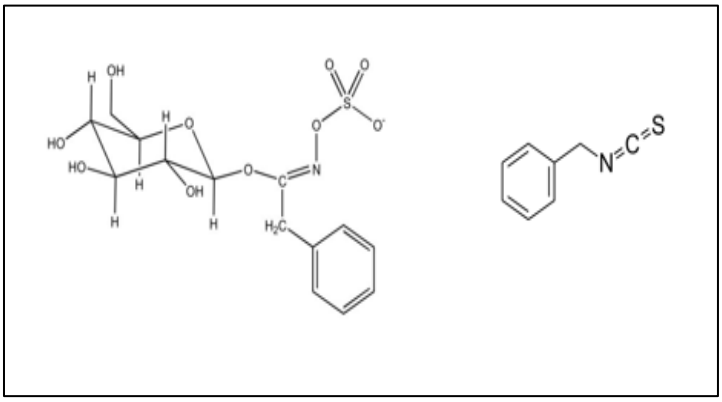

Figure 3. Chemical structures of glucotropaeolin (left) and benzyl isothiocyanate (right) as provided, respectively, by: https://openi.nlm.nih.gov/detailedresult.php?img=PMC548136_1477-7827-3-5-9&req=4 ; By Ed (Edgar181) - Public Domain

More research is needed to understand whether glucosinolates and isothiocyanates are secreted into the soil. Previous research on flavonoids, a class of chemicals to which glucosinolates belong, found that these chemicals are often passively secreted into the soil through decomposing cells or by root cap cells (Weston & Mathesius, 2013). Some evidence suggests that flavonoids may be actively transported and released into the soil, but it is unknown whether this mechanism transports glucosinolates and ITCs. Two experiments (Callaway *et al.*, 2008; Stinson *et al.*, 2006) conditioned soils by growing *A. petiolata* for six and three months, and they found that growing plants suppressed arbuscular mycorrhizal fungi (AMF). These two studies showed that living plants deposit enough allelochemicals to affect AMF; it is therefore quite possible that the effects documented were the result of glucosinolates, and perhaps other flavonoids, secreted into the soil.

Alliaria petiolata's production of glucosinolates is influenced by multiple factors, including whether *A. petiolata* is growing near, and competing with, other plants (Lankau, 2010). When *A. petiolata* was competing with *Platanus occidentalis* (sycamore), it produced significantly greater amounts of both allyl and benzyl glucosinolates in unsterilized soils. This trend was seen in individuals from different *A. petiolata* populations. These findings support the hypothesis that glucosinolates are involved in allelopathy since they were produced in greater amounts in competition, and higher amounts of glucosinolates were associated with lower *P. occidentalis* biomass.

Another factor influencing the production of glucosinolates is population age. Lankau *et al.* (2009) demonstrated different populations of *A. petiolata* produced

different amounts of allelochemicals. Genetic studies were compared to allelochemistry studies and referenced to the length of time since invasion (Lankau *et al.*, 2009). Individuals within old populations (where the population had been present for 50+ years) produced fewer allelochemicals compared to those that had more recently invaded, but populations with the same invasion age produced similar levels of these allelochemicals. Analyses showed that genetic differences were not linked to allelochemical difference. Lankau *et al.* (2009) speculated that, over time, evolutionary pressures change, and allelopathy traits that were beneficial during early invasion became less advantageous in intraspecific competition.

To understand *A. petiolata*'s allelopathy, multiple studies have studied the half-lives of glucosinolates and ITCs. Unfortunately, these studies have confounding results. One study measured the half-lives of sinigrin, 40 days, and allyl ITC, 120 days, derived from *Brassica juncea* (Tsao *et al.*, 2000). In separate studies, researchers found that glucotropaeolin and benzyl ITC have far shorter, and unfortunately variable, half-lives ranging from 4.25 hours to 9 days (Gimsing *et al.*, 2009; Gimsing *et al.*, 2007). However, these studies used chemicals that were derived from a different plant, or from reagents. Studies that have focused on these chemicals from *A. petiolata* have conflicting results.

One study confirmed that AITC and sinigrin from *A. petiolata* are released into the soil, but the length of time that they remained there is unknown (Cantor *et al.*, 2011). Barto and Cipollini (2009) attempted to calculate the half-lives of compounds derived from *A. petiolata* in soils kept in the laboratory, but could not detect the compounds. Neither allyl ITC or benzyl ITC were reliably detected in the soil

samples (Barto & Cipollini, 2009). Sinigrin and glucotropaeolin were not detected in field soils inhabited by *A. petiolata*. They did not test for AITC and BITC in their field soils, so it possible that sinigrin and glucotropaeolin had been converted to these forms. No studies have resolved what factors led to the different allelochemical detection results.

Alliaria petiolata's allelopathy may not only require glucosinolates and isothiocyanates as they are not the only unique compounds in *A. petiolata*. Other novel compounds found in *A. petiolata* include alliarinoside (Haribal *et al.*, 2001) and isovitexin-6"-β-D-glycopyranoside (Haribal & Renwick, 1998), both of which have only been reported in *A. petiolata*. *Alliaria petiolata* also produces relatively high amounts of hydrogen cyanide (Cipollini & Gruner, 2007). Studies have successfully isolated these compounds from tissues, but little is understood about their role or their effect on soil organisms.

Cipollini and Gruner (2007) found that three-week-old seedlings of *A. petiolata* produced 108.9 ppm hydrogen cyanide on average, whereas three other 3-week-old *Brassica* species produced less than 1 ppm of hydrogen cyanide. They also found that *A. petiolata* increased production of cyanide between one and eight weeks, whereas another cyanide producing plant *Sorghum sudanense* decreased cyanide production over time. However, Cipollini and Gruner (2007) did not find the substrates and enzymes involved in cyanide production, and no other published studies to date have found the substrate or process responsible for hydrogen cyanide production in *A. petiolata*.

Haribal (2001) reported finding a novel compound within *A. petiolata* foliage that was named alliarinoside which has, thus far, not been found in any other plants (Figure 4) (Barto *et al.*, 2010; Cappuccino & Arnason, 2006; Haribal *et al.*, 2001). Some evidence shows that alliarinoside functions as an herbivore deterrent in leaves (Cipollini *et al.*, 2005). One study found that as the concentration of alliarinoside in roots increased there was a slight decrease in overall fungal community richness (Lankau, 2011a). Alliarinoside has only been found in roots or leaves, but not soils (Barto & Cipollini, 2009; Haribal & Renwick, 2001; Lankau, 2011a). When alliarinoside was added to soil, it was not detected after 30 minutes, suggesting that it has a very short half-life. It is possible that alliarinoside itself is not allelopathic, but it is produced when other allelochemicals are, or it is the substrate for an allelochemical. Barto and Cipollini (2009) suggested that it could be the source for hydrogen cyanide in *A. petiolata* because it contains a CN side group (Figure 4), but no studies have supported this assertion.

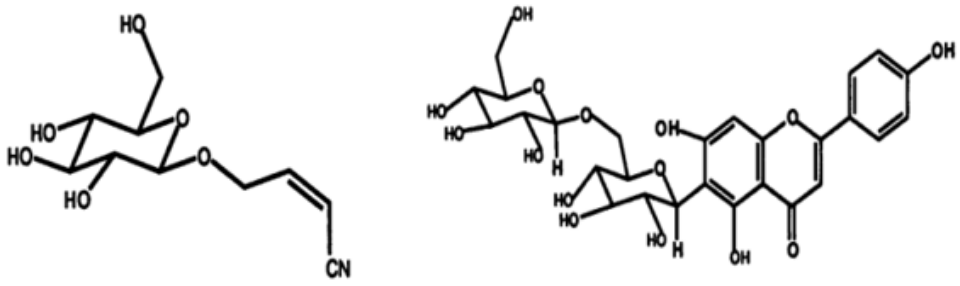

Figure 4. Left: Chemical structure of alliarinoside as shown in Haribal and Renwick (2001). Right: Chemical structure of isovitexin-6”-β-D-glycopyranoside as seen in Haribal and Renwick (2001)

Haribal and Renwick (1998) found another feeding deterrent in *A. petiolata* tissue: isovitexin-6”-β-D-glycopyranoside (isovitexin, Figure 4). Isovitexin was also

detected in the soil, though the release mechanism and possible roles are unknown (Barto & Cipollini, 2009; Haribal & Renwick, 1998). Barto and Cipollini (2009) reported that while they found isovitexin in the soil, it was not produced in root tissues. Studies show isovitexin acts primarily as a feeding deterrents, and not as an allelochemical (Haribal *et al.*, 2001).

Allelopathy Case Studies

Alliaria petiolata's allelopathy affects numerous organisms, including plants. Reduced germination and growth has been documented in both agricultural and forest understory plants. Vaughn and Berhow (1999) found that radicle elongation in *T. aestivum* and *L. sativum* was reduced when allyl ITC, benzyl ITC, sinigrin or glucotropaeolin was added to agar. Sinigrin reduced radicle elongation in only cress, but allyl ITC reduced average radicle length for both species. Both glucotropaeolin and BITC were correlated with reduced radicle elongation. Basil (*Ocimum basilicum*) and lettuce (*Lactuca sativa*) seed germination was inhibited by aqueous *A. petiolata* leachate by ~ 65 and ~35 %, respectively (Cipollini *et al.*, 2012). These leachates also inhibited broccoli germination (*Brassica olearacea*) with the percentage of inhibition being far lower compared to that of basil and lettuce.

Two studies found reduced germination of forest understory herbs. *Geum urbanum* and *Geum laciniatum*, two herbaceous plants native to North America, had germination decreased in *A. petiolata* conditioned soils (Prati & Bossdorf, 2004). A later study looked at three more North American plants, but also compared the effects of root and shoot tissue leachates on germination. *Alliaria petiolata* leachates suppressed the germination of three North American plants *Blephilia hirsute* (hairy

pagoda plant), *Elymus hystrix* (bottlebrush grass), and *Anemone virginiana* (tall thimbleweed). Both root and shoot leachate suppressed germination, but far fewer plants germinated when treated with shoot leachate.

Effects of *A. petiolata* can also be modulated by the microbial community surrounding a plant. Lankau (2010) studied the effect of *A. petiolata* on sycamore (*Platanus occidentalis*) when grown in sterile vs. non-sterile soil. In sterile soil, *A. petiolata* presence reduced *P. occidentalis* biomass. In non-sterile soil, *A. petiolata*'s presence did not reduce biomass as much as it did in sterile soil (Lankau, 2010). These results show that microbial communities could modulate the effect of *A. petiolata* allelopathy.

Roberts and Anderson (2001) found that *A. petiolata* also affects mycorrhizal relationships between plants and fungi. The experiments showed that *A. petiolata* leachate ceased colonization of arbuscular mycorrhizal colonization in tomato (Roberts & Anderson, 2001). Seedlings grown in media containing *A. petiolata* leachate had shorter roots than those in the control (2.91 ± 0.76 cm vs. 8.05 ±1.88 cm). This study became the basis for further mycorrhizae-*A. petiolata* studies.

Stinson *et al.* (2006) found that *Alliaria petiolata*'s impacts on mycorrhizal fungi affected forest trees. Soils were treated by having one of three native tree species (red maple, *Acer rubrum*, sugar maple, *A. saccharum*, or white ash, *Faxinus americana*) or *A. petiolata* growing for three months. The treatment plants were removed and the soils were used to grow one of the three tree species previously used. After four months, the roots were collected and assessed for mycorrhizal colonization. Trees grown in *A petiolata* treated soils had between 0% to 10%

mycorrhizal colonization in roots. In soils treated with native tree species, mycorrhizal colonization ranged from 20% to 65%. These results demonstrated how suppression of the mycorrhizal community prevented the growth and survival of plants dependent upon arbuscular mycorrhizal fungi, particularly canopy trees (Stinson *et al.*, 2006). Similarly, *A. petiolata* decreased AMF infectivity in *Acer rubrum*, though several AMF taxa were still present (Barto *et al.*, 2011). When *A. petiolata* was present in the environment, roots were colonized with fewer AMF hyphae and arbuscules depending on site. This study supported previous findings that AMF colonization changes when *A. petiolata* is in an environment.

In their 2006 study, Stinson *et al.* predicted that plant communities would change because mycorrhizae were suppressed. The predictions were supported when another study showed that *A. petiolata* abundance was negatively correlated with species evenness in forest environments (Stinson *et al.*, 2007). This study looked at whether *A. petiolata*'s presence, or eradication, affected functional group diversity in forest understory vegetation and how functional groups responded to *A. petiolata* presence. Vegetation censuses were conducted at sites with varying levels of *A. petiolata* cover twice within one summer, and once the following summer. After the census, species were sorted into functional groups. The relative abundance of different functional groups, and relative abundance for each species was measured. Shannon's equitability and Shannon's diversity index declined as *A. petiolata* cover increased. In another experiment, sites with 30-35% *A. petiolata* cover were selected for eradication experiments; *A. petiolata* was partially or fully removed from plots and a plant census was conducted one year later. Equatibility and diversity increased

in plots where all *A. petiolata* was removed, but these numbers did not change in plots where *A. petiolata* was only partially removed. Not all functional groups were negatively affected by *A. petiolata* presence. The relative abundance of tree seedling and graminoid was lower when *A. petiolata* cover was higher. These results supported the predictions from the 2006 study and showed that *A. petiolata*'s presence was correlated with reduced plant diversity.

Callaway *et al.* (2008) showed that *A. petiolata*'s effect on AMF and plants is strongest on species from North America. Arbuscular mycorrhizal fungi grown in soils from North America and Europe that were conditioned with *A. petiolata* had lower density, viability, and infectivity. *Alliaria petiolata* was consistently correlated with decreased viability and infectivity of North American AMF spores (-20 to -60% and -16 to 68%, respectively) (Callaway *et al.*, 2008). *Alliaria petiolata* soil conditioning also affected the biomass of native plants. Plants native to North America were more likely to have lower biomass in *A. petiolata* conditioned soils. Plants native to Europe did not show any consistent suppression by *A. petiolata* conditioning. This study suggests that *A. petiolata* is consistent the Novel Weapons Hypothesis.

One study found that the concentration of *A. petiolata* allelochemicals detected in soils suppressed AMF germination *in vitro*. Allyl ITC was found in soils inhabited by *A. petiolata* at a concentration of 0.001 mmol AITC/gram of soil, and soils with no *A. petiolata* had no detectable allyl ITC (Cantor *et al.*, 2011). At 0.001 mmol/gram, *Glomus clarum*, an AMF species, germination decreased *in vitro*. This

study strengthened the case for *A. petiolata*'s allelopathy by demonstrating that the concentration of *A. petiolata*'s allelochemicals in the soil suppress AMF germination.

Cantor *et al.* (2011) also compared the overall density and length of hyphae in soils that were invaded versus those that were uninvaded by *A. petiolata*. Soils where *A. petiolata* was had a 37% lower abundance of hyphae (Cantor *et al.*, 2011). While the species of fungi were not identified or what their ecological role was, *A. petiolata*'s presence affects many fungi in the soil.

In another study, Barto *et al.* (2010) studied the effects of flavonoids and glucosinolates derived from *A. petiolata* on germination, pre-symbiosis growth, symbiosis formation, symbiosis growth, and symbiosis in jewelweed (*Impatiens pallida*). The combined flavonoid and glucusinolate fraction decreased germination. The combined treatment also stunted the growth of roots in pre-symbiosis plants and shortened their life spans. The glucosinolate only treatment decreased the life span of *I. pallida* individuals, but did not affect their biomass before they died. When *I. pallida* plants were introduced to symbiotic AMF, *A. petiolata* extracts did not affect growth and biomass, but it did affect colonization.

Barto *et al.*'s (2010) work showed that *A. petiolata* affected specific early life stages, specifically germination and pre-symbiosis growth. Both Stinson *et al.* (2006) and Callaway *et al.* (2008) conditioned soils with *A. petiolata* and then measured AMF and plant growth, but these studies used plants that had not yet formed symbioses. Callaway *et al.* (2008) also found that *A. petiolata* lowered AMF spore viability, which could explain suppressed plant growth and AMF colonization. Barto *et al.* (2010) indicated that trees and other plants with symbioses will not be affected

by *A. petiolata*. This conflicts with Stinson *et al.*'s (2007) study that found that canopy tree seedlings were less abundant where *A. petiolata* was present.

Some ectomycorrhizal fungi are also affected by *A. petiolata* leachate both in the field and *in vitro* (Wolfe *et al.*, 2008). Tree species in the field that associated with ectomycorrhizal fungi showed lower colonization in root tips. Spores of *Hebeloma crustuliniforme, Laccaria bicolor,* and *Scleroderma cepa* failed to germinate in media treated with benzyl ITC added to it in contrast to control plates in which germination and growth were normal. The fungi used in the germination experiment are facultatively mycorrhizal. More studies are necessary to understand what percentage of ectomycorrhizal fungi and plants have similar effects. Thus, it is possible that canopy trees with EMF relationships will have lower colonization, and less access to nutrients. This is the only study on both EMF and facultative mycorrhizal fungi, so it is unknown whether the effects that they documented are common amongst all EMF, or all facultative mycorrhizae. Therefore, little is known about whether being facultatively or obligately mycorrhizal affects likelihood of suppression.

Alliaria petiolata's allelopathy adversely affects more than just mycorrhizal fungi and plant diversity. One study (Lankau, 2011b) demonstrated that soil bacterial, general fungal, and AMF diversity after *A. petiolata* reduced over time. Sites where *A. petiolata* was present for 30 to 45 years had less diverse microbial communities than sites where *A. petiolata* was present for less than 30 years or more than 45 years. Arbuscular mycorrhizal fungal diversity decreased the most. Soils were also tested for presence of susceptible taxa by measuring soil microbial diversity before and after *A.*

petiolata was planted in that soil. Where *A. petiolata* had invaded less than 30 years and 47 to 50 years earlier still had taxa sensitive to the plant's leachates. Soils invaded 30 to 45 years before had very few susceptible taxa. This study demonstrated that while most North American soil microorganisms are susceptible, some are resistant to *A. petiolata* allelopathy.

While multiple studies have documented adverse effects on fungi from multiple phyla, we do not know how *A. petiolata* affects all fungi. For example, effects on sought-after edible fungi are poorly understood. In North America, one popular edible that has not been formally studied in relation to *A. petiolata* invasion is *Morchella*, the morel mushroom.

Research Objectives

Morchella is a genus with many edible species and is highly sought after in North America. Species are found in many areas, but are generally endemic to either the east or west with the Rocky Mountains as the barrier (O'Donnell *et al.*, 2011). At least two species in this genus will form ectomycorrhizae, including a mantle and Hartig net, with American elm (*Ulmus americana*), slippery elm (*U. rubra*), apple (*Malus sylvestris*), and black spruce (*Picea mariana*) and transfer nutrients from substrate to host (Harbin, 1999). A follow-up study found that *M. "esculenta"* and *M."rotunda"* sometimes formed ectomycorrhizae with three tree species (western larch, *Larix occidentalis*, lodgepole pine, *Pinus contorta*, and Douglas fir, *Pseudotsuga menziesii*) (Dahlstrom *et al.*, 2000). No experiments have confirmed that these associations occur in nature, or whether mycorrhizal associations are common throughout the genus.

Multiple *Morchella* species live in habitats that *A. petiolata* invades. No published data demonstrates whether *Morchella* distribution or fruiting is affected. One anecdotal observation (TJV, pers. Comm.) suggested that black morels (*M. angusticeps*) stopped fruiting following the introduction of *A. petiolata* at one site in south central Wisconsin. The black morels had fruited under black cherry trees (*Prunus serotinia*) every year prior to *A. petiolata*'s introduction but stopped after introduction. No experiments or studies determined whether *A. petiolata* or another factor caused the morels to stop fruiting at that site.

This study explores whether *Morchella* growth is affected by *A. petiolata*. Based on *Alliaria petiolata*'s proven broad spectrum allelopathic effects and on reduced morel fruiting after *A. petiolata* introduction in the field, we hypothesize that *Morchella*'s growth is negatively affected by aqueous leachates of *A. petiolata*. *In vitro* tests were conducted on *Morchella* cultures using aqueous leachates of whole *A. petiolata* plants as well as leachates of *A. petiolata* roots alone and shoots alone so that each tissue could be tested separately for effects on *Morchella* growth.

CHAPTER II

IN VITRO EXPOSURE OF *MORCHELLA* TO *ALLIARIA PETIOLATA* LEACHATE

The invasive plant *Alliaria petiolata* (garlic mustard, Brassicaceae) is known to suppress the growth of mycorrhizal fungi, and by extension, the plants that rely on these fungi (Callaway *et al.*, 2008; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). Numerous studies have investigated *A. petiolata*'s effects on arbuscular mycorrhizal fungi, and one study looked at a few ectomycorrhizal fungi (Callaway *et al.*, 2008; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). Despite these efforts, there are still many taxa of mycorrhizal fungi that have not yet been studied. The evidence reported thus far suggests that other fungal taxa are probably susceptible to *A. petiolata* negative allelopathy, but studies are needed to understand the percentage and types of fungi affected by this plant. There is currently no evidence for whether edible mycorrhizal fungi are affected by *A. petiolata*.

Alliaria petiolata's invasion success is often attributed to the Novel Weapons Hypothesis (Callaway & Ridenour, 2004) because it suppresses mutualistic soil organisms, and the plants that rely on them, through allelopathy. An introduced plant produces an allelopathic chemical (or allelochemical) to which native plants are naïve and thus more susceptible, as compared to organisms that coevolved with the introduced plant.

In *A. petiolata*, several compounds have been isolated and tested for the ability to suppress soil microorganisms including arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) (Callaway *et al.*, 2008; Portales-Reyes *et al.*, 2015; Stinson et al., 2006; Wolfe et al., 2008). Sinigrin and glucotropaeolin, which are converted by myrosinase enzymes to allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC) respectively have been the most intensively studied as allelochemicals (Callaway *et al.*, 2008; Vaughn & Berhow, 1999; Wolfe *et al.*, 2008). Other compounds that potentially play a role in allelopathy are alliarinoside (Lankau, 2011a), isovitexin-6”-β-D-glycopyranoside (isovitexin) (Haribal & Renwick, 2001) and hydrogen cyanide (Cipollini & Gruner, 2007). Glucotropaeolin and BITC are only found in root tissues, and isovitexin has only been isolated from shoot tissues, alliarinoside and sinigrin/allyl isothiocyanate are found in both tissue types (Barto & Cipollini, 2009). Yet, there is little information on how they are released into the soil.

Studies suggest that *A. petiolata* allelochemicals significantly alter the soil microbial communities and that these effects will last for years (Lankau *et al.*, 2014). Six years after *A. petiolata* eradication, Lankau *et al.* (2014) demonstrated that AMF diversity and richness was similar to control plots with no removal. Plots with lower densities of *A. petiolata* prior to removal were more likely to regain some AMF diversity. Unfortunately, no uninvaded sites were sampled or studied to compare to removal and control plots. Previous studies found that in two sites, the diversity of AM fungi associated with *Acer saccharum* did not decrease, but colonization and AM structures decreased (Barto *et al.*, 2011). Lankau *et al.* (2014) showed that density of AM fungi only increased under certain conditions.

The body of evidence suggests that *A. petiolata* suppresses a broad spectrum of organisms, such as arbuscular- and ecto- mycorrhizal fungi (Callaway *et al.*, 2008; Lankau, 2011b; Portales-Reyes *et al.*, 2015; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). However, some organisms are resistant to garlic mustard's allelochemicals (Lankau, 2011b). There are still many species of fungi and other organisms that have not been studied for resistance or susceptibility to *A. petiolata*.

Morchella is a beloved genus of fungus that are sought after in North America. There are multiple species that belong to either the Esculenta clade (yellow morels) or Elata clade (black morels) (Kuo *et al.*, 2012). Morels are Ascomycetes that are found globally but have high endemism to specific regions (O'Donnell *et al.*, 2011; Richard *et al.*, 2015). In North America, most species are usually found in either the East or the West (divided by Rocky Mountains). Although commonly harvested by mushroom hunters, but their exact distribution and population size remains poorly understood.

A laboratory study by Harbin and Volk (1999) showed that *M. esculenta* and *M. angusticeps* can form ectomycorrhizae with a few plant hosts. *Morchella esculenta* (Figure 5) formed mycorrhiza with *Malus sylvestris*, *Ulmus americana*, and *Ulmus rubra* seedlings. *Morchella angusticeps* (Figure 5) formed mycorrhizae with black spruce (*Picea mariana*). It is likely that *Morchella* forms mycorrhizae in nature because it is commonly associated with *U. americana* and *M. sylvestris*. Previous studies have found that *Morchella* can grow saprotrophically on nutrient media and in grain spawn, so *Morchella* are not obligate mycorrhizae (Volk & Leonard, 1989).

However, it is unknown if this association occurs in nature or whether it is necessary for *Morchella*'s life cycle.

Multiple *Morchella* species are found in forests, usually associated with one or more tree species, where *A. petiolata* has invaded or is likely to invade. Field data (TJV, pers. comm.) suggest that black morels stopped fruiting below living black cherry trees (*Prunus serotinia*) in south central Wisconsin shortly after *A. petiolata* entered the site. The present study attempts to discover how *Morchella* responds to *A. petiolata* by studying the effect of aqueous whole plant, shoot, and root leachates on *Morchella* characteristics *in vitro*.

Figure 5. Left *Morchella americana* from http://mushroomobserver.org/204019 Right: *Morchella angusticeps* from http://www.mushroomexpert.com/images/kuo/morchella_angusticeps_02.jpg

Materials and Methods

Morchella Sampling and Culturing

Morchella cultures represented both the yellow clade (Esculenta) and the black morel (Elata). All yellow samples were collected in southwestern Wisconsin in the spring of 2015. All specimens appeared to belong to the species *Morchella americana,* although it is possible one or more belonged to a cryptic species *Morchella ulmaria* that is differentiated by genetics and not morphology (Richard *et al.*, 2015). Black morel spore samples were collected around the country in Spring 2015 by volunteers contacted over the internet. The black morel samples were sent from New York, Connecticut, Quebec, and Wisconsin. The samples' morphology and geographic origin corresponds to *M. angusticeps*.

Table 1. Site data for isolates used in the study.

Sample Code	Species	Location	Habitat
M.a. 1	*M. angusticeps*	Poughkeepsie, NY	NA
M.a. 2	*M. angusticeps*	Poughkeepsie, NY	NA
M.a. 3	*M. angusticeps*	Trumbull, CT	NA
M.a. 4	*M. angusticeps*	Trumbull, CT	NA
M.a. 5	*M. angusticeps*	Highland Mills, NY	NA
M.a. 6	*M. angusticeps*	Wittenberg, WI	Sandy, near *Pinus strobus*, *Populus* sp., *Alnus sp.*
M.a. 7	*M. angusticeps*	Wittenberg, WI	Sandy, near *Pinus strobus*, *Populus* sp., *Alnus sp.*
M.a. 8	*M. angusticeps*	Pierre Fonds, Quebec	NA
M.a. 9	*M. angusticeps*	Newport State Park, WI	Near *Larix* sp.
CEF	*M. americana*	Coulee Experimental Forest, La Crosse WI	Near *Ulmus americana*
HIX	*M. americana*	Hixon Forest, La Crosse, WI	Near *U. americana*
BL	*M. americana*	Mathy Property, La Crosse, WI	Near *U. americana*

Morchella fruiting bodies were placed in Petri dishes or on aluminum foil to collect spores. The spores were collected by pipetting a small amount of sterile water onto the spore deposit to create a suspension that was pipetted onto a potato dextrose agar (PDA) plate. A sterile plate spreader was used to separate as many spores as possible. The plate spreader was then also applied to a second PDA plate to dilute the spores and prevent plasmogamy. Twenty-four hours later, germ tubes were found using a Nikon Eclipse E400 stereoscope. Single spore isolates were transferred to separate PDA plates using a scalpel. Eight black morel single spore isolates and seven yellow morel isolates were used in the experiment.

A code was developed to identify different cultures (Table 1). The code is based on three factors: the clade that the culture belonged to, a number assigned to the parental fruiting body, and then a number based on how many cultures were obtained from that parent. For *M. americana* cultures, the locality of parent culture was a factor. The experimental design called for six replicates each of the five treatments. However, some replicates became contaminated and those plates were not included in analysis. The total number of black morel replicates was 186 and the total number of yellow morel replicates was 163.

Leachate Preparation

Leachates were derived fron *A. petiolata* basal rosettes collected from a roadside (1-1.5 m from road) in Vernon County, WI in mid-September. In the lab, most dirt was removed and washed away. The plants were randomly sorted to be made into different leachates: whole plant, shoots, and roots. The plant materials were soaked in water without shaking at a concentration of 220 g plant material per liter of

water for approximately 18 hours at room temperature. The plant material was filtered in a sequence of >8 μm, 8 μm, 2.7 μm, and 0.1 μm pore size to remove plant and bacterial cells. Leachates were not autoclaved, or otherwise sterilized to preserve the leachate's composition. The leachate was stored in a freezer at -20º C to prevent contamination and to slow any chemical or biological degradation of the leachate. The leachates were completely thawed before being used in the experiments.

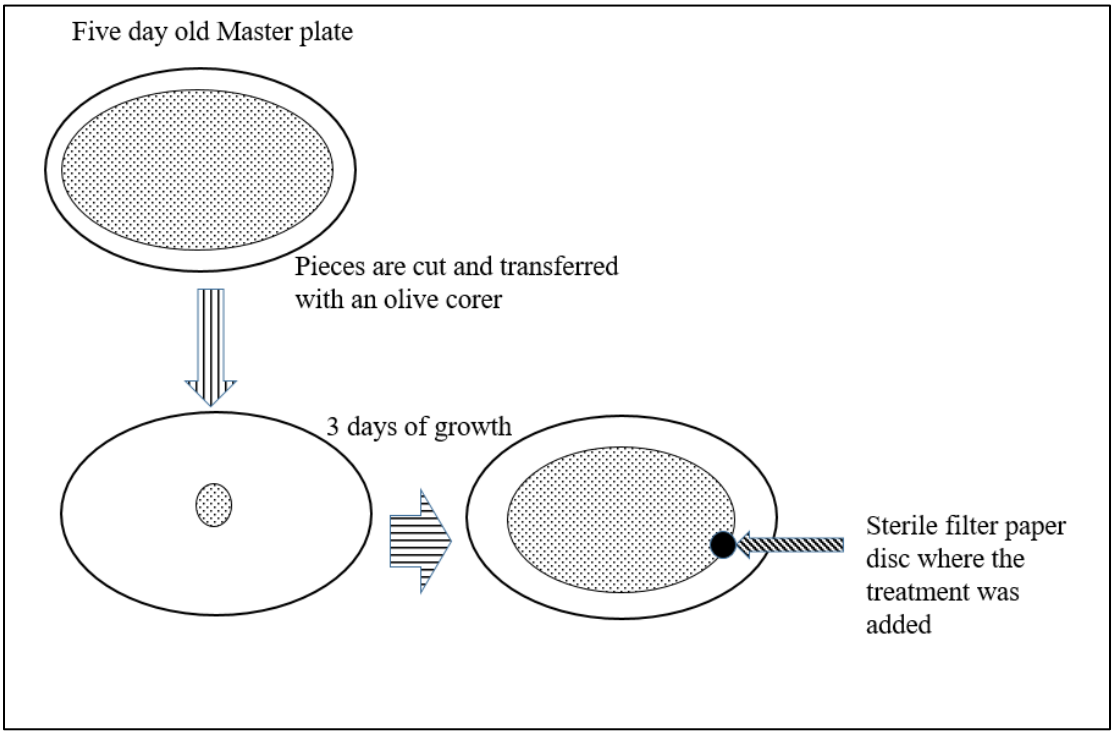

Figure 6. Diagram illustrating the transfer of cultures from master plates, to treatment plates, and then adding treatment.

The positive control was an aqueous solution of allyl isothiocyanate and benzyl isothiocyanate. Allyl isothiocyanate was diluted to 0.001 μM and BITC was diluted to 7.5 nM (Cantor *et al.*, 2011; Wolfe *et al.*, 2008).

Experimental Design

Cultures used in these experiments were first grown on master plates for five days on PDA. After five days, plugs of mycelium were transferred to half-strength

PDA and allowed to grow for three days. Half-strength PDA was used because changes were more easily observed on lower nutrient media, but the mycelium becomes hard to see if the nutrients are too low. A sterile 5 mm circle of filter paper was placed near the growing edge of the mycelium. One hundred μl of root, whole plant, shoot, water, or ITC treatment was added to the growing margin so that it would interact with an actively growing portion of the culture (Figure 6).

The plates were placed under a Nikon SmZ645 dissecting scope, and photographed at 8x magnification with a Nikon E995 camera approximately every twenty-four hours for seven days. The photos were analyzed for four different characteristics: mycelial deformation, line production, pigment production, and zone of inhibition (see Results, Figure 7). Each trait was assessed as either absent (0) or present (1).

The distribution of presence/absence in each trait was compared between the experimental leachate treatment and the negative water control. Each trait in each treatment was compared within each treatment.

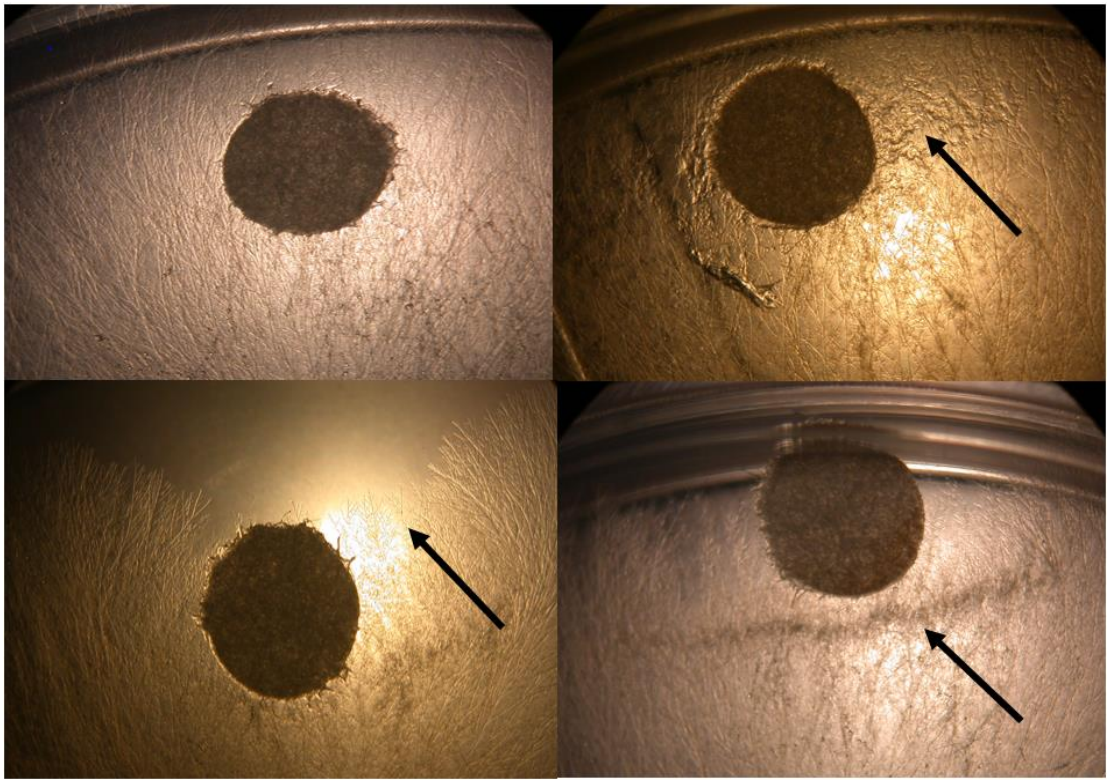

Figure 7. Clockwise from top left: An unchanged, normal culture after 24 hrs, deformation trait after 24 hrs, line and pigment trait after 24 hrs, zone of inhibition trait after 24 hrs.

Statistical Analysis

The counts of 1s and 0s were used to analyze the difference between control and each experimental treatment for each day. The counts were placed into matrix used to analyze the distribution/proportion of 1's and 0's. Data were analyzed with a Chi-squared test for independence when the cell counts were greater than five, or Fisher's exact test when cell counts were five or fewer. By comparing these distributions, it helped illustrate whether the responses to *A. petiolata* leachate were significantly different from sterile water. Because many tests were conducted, a Bonferroni correction for multiple tests was applied to the p-values, and these values were used to determine whether results were statistically significant.

Results

On average, black and yellow morels responded differently depending on trait and treatment (Figure 8). One leachate caused both black and yellow morels to exhibit the same response, but often in different proportions. Traits would often appear or disappear in cultures as the mycelium developed.

These trends are illustrated by the average proportion of 1s over seven days for each treatment and trait. When given the whole plant leachate more black morel cultures expressed the deformation trait, on average, than yellow morel cultures (Figure 8a). More yellow morel cultures expressed the pigment trait than black morel cultures (Figure 8a). The line trait was expressed in similar averages when given whole plant leachate (Figure 8a). When given root leachate, black morels had higher average responses than yellow morels for all four traits (Figure 8b). Yellow and black morel cultures given shoot leachate expressed the pigment trait in similar average proportions, but they expressed the deformation and line traits in different proportions (Figure 8c). Black morel cultures given shoot leachate expressed the deformation trait more, on average than yellow morel cultures (Figure 8c). Yet, the shoot leachate elicited the line trait in more yellow morel cultures than black morel cultures (Figure 8c).

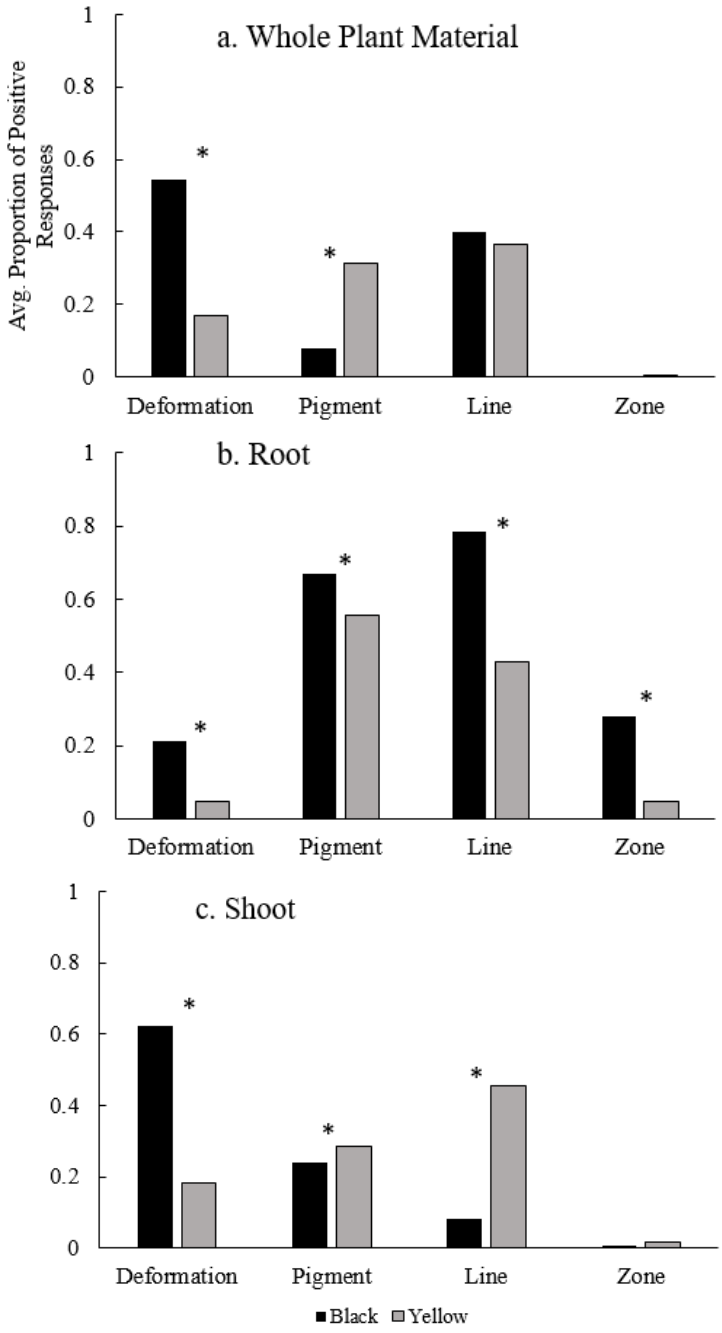

Figure 8. The average proportion of positive responses over 7 days for each treatment. a: Whole Plant, b: Roots, c: Shoots. (black morels n=186, yellow morels n=163) *=p<0.01

Deformation Trait

The deformation trait was common in both black and yellow morel cultures, but it was also very common in cultures treated with water. Across all seven days the proportion of black cultures given water was between 0.69 and 0.64 (Figure 9a).

Among black cultures, more than half of those given shoot leachate exhibited this trait across all seven days (0.83 on Day 1). When given whole plant leachate, more than half of cultures exhibited this trait (0.62 on Day 1), but this dropped over time (0.4 on Day 7). However, black cultures given root leachate exhibited this trait in a slightly lower proportion on the first day (0.48 on Day 1), and this trait disappeared from most cultures over time (0.02 on Day 7) (Figure 9a). In these cultures, as the culture aged and the mycelium developed, the deformation trait, and others, disappeared. On the last three days, so few black cultures treated with root leachate exhibited this trait that it was significantly different from the water treatment ($p<0.01$).

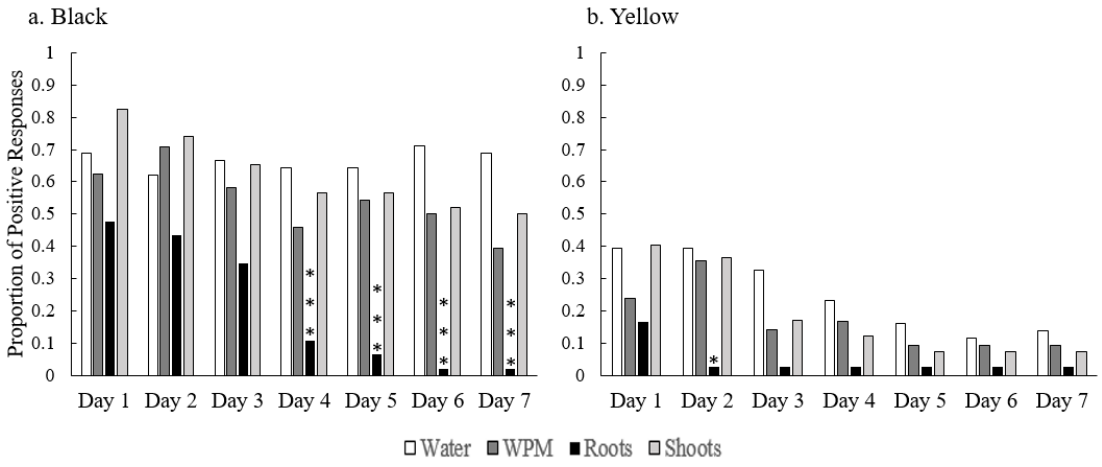

Figure 9. Proportion of positive deformation responses over time for both black and yellow morels. a: Black morels, b: Yellow morels (n=186 for black morels, n=163 for yellow morels). *=p<0.05, **=p<0.01, ***=p<0.001

All yellow morel cultures exhibited the deformation trait in lower proportions than black morels. However, within the yellow morels, those given water or shoot leachate had the highest proportions of deformation trait (0.4 on Day 1 for both) (Figure 9b). Over time, fewer cultures given shoot leachate exhibited this trait than those given water (0.07 on Day 7 for shoot leachate, and 0.14 for water). Those given

whole plant leachate exhibited this trait in slightly lower proportion. The cultures given root leachate had the lowest proportion (0.17 on Day 1), and, like the black cultures, this trait tended to disappear from the mycelium over time (0.03 on Day 7) (Figure 9b).

Line Trait

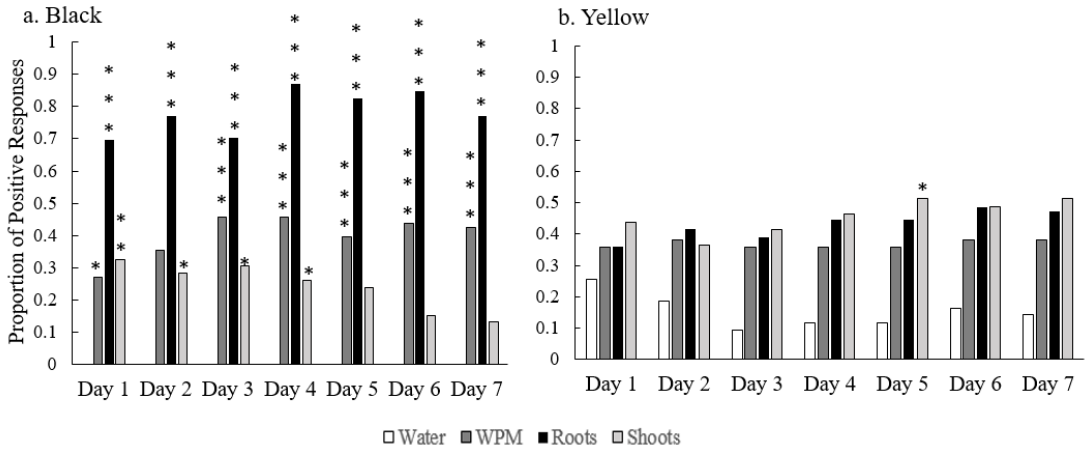

Figure 10. Proportion of positive line responses in both black and yellow morels over time. a. Black morels (n=186) b. Yellow morels (n=163), *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$

The line trait was somewhat common amongst both black and yellow morels, especially those given root leachate. For black morels, the line trait appeared in between more than half of the cultures given root leachate (0.70 on Day 1), and the number of cultures exhibiting this trait increased slightly over time (0.77 on Day 7) (Figure 10a). A similar trend was seen in black cultures given the whole plant leachate, but the proportions were lower (0.27 on Day 1, 0.43 on Day 7). The line trait appeared in a significant number of cultures given the shoot leachate on the first day, but this trait disappeared over time (0.33 on Day 1, 0.13 on Day 7) (Figure 10a). The line trait was never significant in the shoot treatment beyond the first day.

Yellow morel cultures exhibited the line trait, but in very different treatments. Some cultures in the water control also expressed the line trait (Figure 10b). Among the leachates, the line trait was exhibited in close proportions, with shoot leachate having the highest proportion most days (0.44 on Day 1 and 0.51 on Day 7). However, the proportion of cultures expressing the line trait increased very slightly in the shoot and root leachate (Figure 10b). Yellow cultures treated with whole plant leachate that exhibited this trait remained nearly constant over time (0.36 on Day 1, 0.38 on Day 7).

Pigment Trait

Both yellow and black cultures exhibited the pigment trait, but in very different proportions, depending on the treatment. This trait was very common among black cultures given the root leachate (0.5 on Day 1, and 0.76 on Day 7), and very rare in cultures given either whole plant or shoot leachate (0.02 for both on Day 1, 0.09 for whole plant and 0.13 for shoot on Day 7) (Figure 11a). On all seven days, a significant number of cultures treated with root leachate exhibited this trait ($p<0.01$) and was never significant for neither whole plant nor shoot leachates. This trait developed in more cultures over time, in all leachates, and even some cultures given water on the last three days (0.02 on Days 5, 6, 7) (Figure 11a).

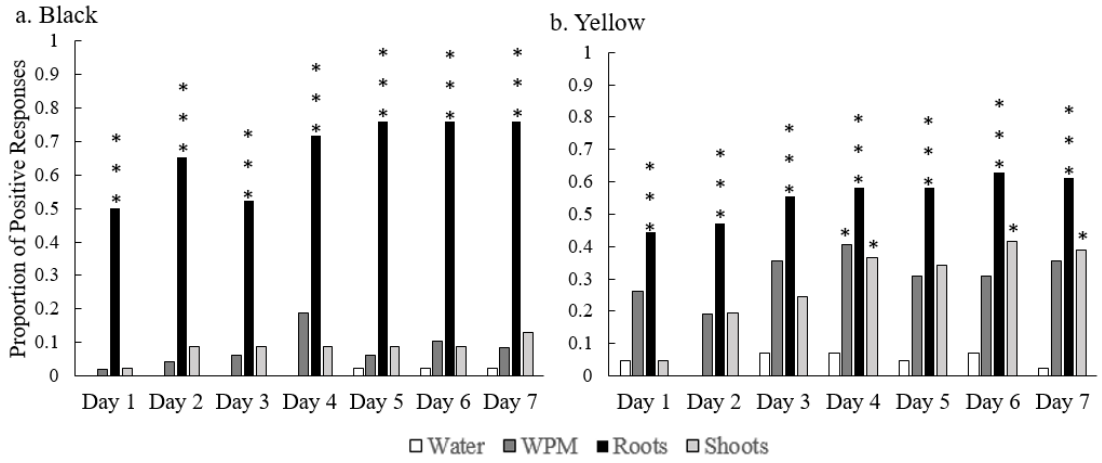

Figure 11. The proportion of positive pigment responses in both black and yellow morels over time. a. Black morels (n=186), b= Yellow morels (n=163) *=p<0.05, **=p<0.01, ***=p<0.001

The pigment trait was exhibited in fewer yellow cultures given the root leachate (as compared to black), but in more cultures given the whole plant or shoot leachates (Figure 11b). A small number of control cultures exhibited the pigment trait (0.05 on Day 1 and 0.02 on Day 7). Less than half of yellow cultures given root leachate exhibited this trait on the first day (0.41), but that increased to over time (0.61) (Figure 11b); the proportion of cultures exhibiting this trait was significant on all seven days ($p<0.01$). Yellow cultures given whole plant or shoot leachate tended to develop this trait over time. The proportion of cultures exhibiting the pigment trait in these treatments was low in the beginning, but rose over time (Figure 11b). The number of cultures given shoot leachate was only significant on days six and seven ($p<0.05$). The number of cultures given whole plant leachate was significant on day four ($p<0.05$).

Zone of Inhibition

The zone of inhibition trait was very rare, and it appeared primarily in cultures given root leachate. Both black and yellow cultures expressed this trait, but only

black morels were statistically significant. In the first three days, a significant proportion of black cultures exhibited this trait ($p<0.01$). After three days, some of those cultures overcame the inhibition, as the proportion of black cultures exhibiting this trait dropped from 0.48 on the first day to 0.13 on the fourth day, but a few maintained it over time (0.2 on Day 7) (Figure 12a). A very small number of cultures given whole plant or shoot leachates expressed the zone of inhibition, and they overcame it after the first day.

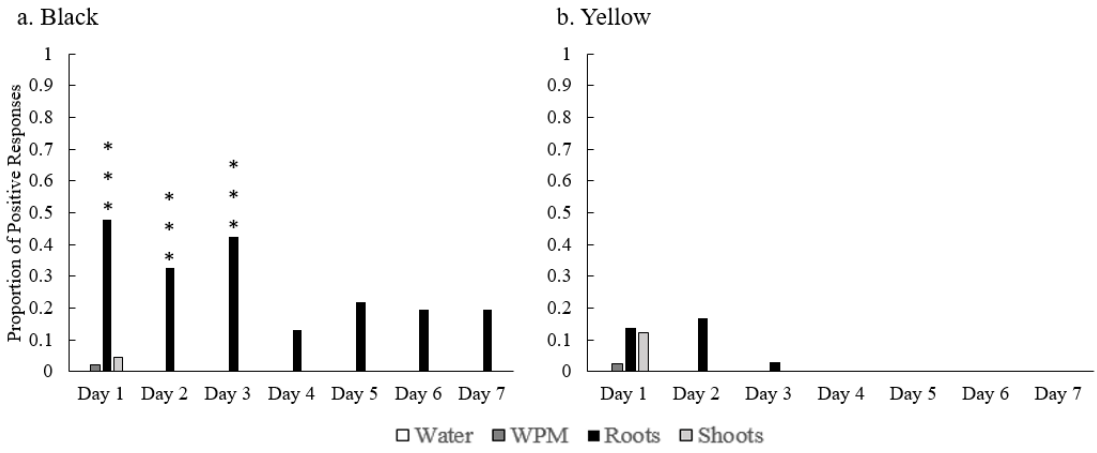

Figure 12. Proportion of positive zone of inhibition responses for both black and yellow morels over time. a. Black morels (n=186), b. Yellow morels (n=163). *=$p<0.05$, **=$p<0.01$, $p<0.001$

In yellow morel cultures, a small number of cultures given root leachate expressed this trait on the first three days 0.14, 0.17, 0.03 respectively (Figure 12b). Fewer yellow cultures given whole plant or shoot leachates expressed this trait, and only on the first day (0.02 for whole plant, and 0.12 for shoot). Yellow cultures never exhibited this trait in significant numbers in all treatments.

Isothiocyanate Control

Reagents of AITC and BITC were prepared and given to one treatment to act as a positive control, to shed light on whether these compounds could be involved in

suppressing *Morchella*. There was very little difference between the reagent treatment and the water control. There were a few cases where the water control elicited a stronger reaction than the isothiocyanate treatment.

Discussion

Alliaria petiolata suppresses multiple mycorrhizal fungi in North America. Evidence has shown that both arbuscular and ectomycorrhizal fungi are affected, but not all species within both groups are susceptible and not all taxa have been studied. For instance, edible mycorrhizal fungi—such as boletes, chanterelles, and morels—have not been investigated. This study aimed to understand how morels responded to *A. petiolata*. Morels are popular edible mushrooms that Harbin and Volk (1999) showed can facultatively form mycorrhizae in greenhouse and laboratory settings, although this has not been demonstrated in nature beyond the observation that most morel species are associated with at least one tree species (Kuo *et al.*, 2012). No published studies have looked at whether any morel species are affected by *A. petiolata*'s presence.

Studying morels in the field is difficult because fruiting is unpredictable. It would be difficult to deduce whether *A. petiolata* affects morel fruiting because there could be other factors affecting fruiting. Thus far, molecular tools have not been tried for detecting *Morchella* in the soil. *In vitro* methods were used to observe changes to *Morchella*, and removed confounding factors that exist in nature.

This experiment studied whether *A. petiolata* affected deformation, pigment production, line production, and zone of inhibition in *Morchella* myclium *in vitro*. This study also compared leachates of different tissues to determine where negative allelopathic chemicals are produced. Pigment production and line production could potentially indicate stress in the mycelium. Zone of inhibition suggests that suppression in nature is possible. The deformation trait appeared in many cultures

given the water control, and it seems that this trait is the result of a large amount of liquid (Figure 13).

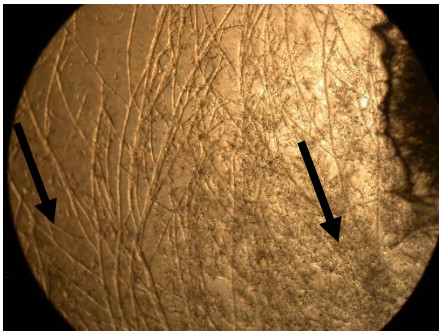

Figure 13. The deformation trait up close. The left arrow is normal mycelium and on the right arrow is mycelium that seems deformed, (more dense).

Two distinct findings came out of this experiment: root leachate was linked to the greatest visible changes, and the black morel cultures (*Morchella elata* clade) were more likely to respond. The root leachate consistently elicited pigment and line traits in both yellow and black morels, and was more likely to elicit a zone of inhibition from black morels. More black morel cultures produced visible responses to the different leachates than yellow morel cultures (Figure 8). Root leachate consistently elicited line production and pigment production in both yellow and black morel cultures, and elicited a zone of inhibition in a significant number of black cultures. Whole plant or shoot leachate rarely elicited significant responses.

Black morels given the root leachate consistently had the strongest responses of all treatments for the line, pigment production, and zone of inhibition. Black morel cultures given either whole plant or shoot leachate only expressed the line trait in significant proportions. Yellow morel cultures did have higher expression of line production and pigment production when given water, or whole plant or shoot leachate than black morel cultures, but expression of traits in these treatments was

rarely significant. Based on these results, black morels seem more susceptible to suppression.

Sample size for this experiment was quite small, cultures were derived from seven yellow morels and eight black morels. The sample size is too small for the results to represent both black and yellow morel populations. Our results can help inform future experiments but cannot be used to generalize about wild populations. Black morels were collected from disparate locations, so they may not represent their respective populations. Yellow morels were all from the same region, so they more likely represent the local population in western WI. Yet, the results show that these morel cultures responded differently depending on their clade. All four traits—deformation, line, pigment, and zone of inhibition—were expressed in both black and yellow morel cultures, but the proportion of cultures expressing each trait varied.

Genetic1 differences between black and yellow morels could account for the different responses. Yellow and black morels could have different traits regulating how they respond to *A. petiolata*'s allelochemicals. Recent analyses revealed that *Morchella* species are endemic to limited geographic regions. *Morchella americana* has been reported exclusively in North America, but close relatives are found in Europe (O'Donnell *et al.*, 2011). *Morchella angusticeps* is also found strictly in North America, and its closest relative is found in China. Unfortunately, nothing is known about *A. petiolata*'s evolutionary history and whether *M. americana* or *angusticep*'s ancestors encountered *A. petiolata*'s ancestors.

The history of garlic mustard invasion could also explain the difference. If the population of yellow morels had previously encountered *A. petiolata* then they could

have developed a resistance to its effects. The history of *A. petiolata* presence or absence is unknown for the different sites. Though *A. petiolata* is found in the regions where morels were sampled.

Comparing Leachates

Root leachates elicited more responses from both yellow and black morels for the pigment and line traits and elicited a zone of inhibition in black morels (Figure 8, 9, 10, 11). This finding fits our current understanding of *Alliaria petiolata* as an allelopathic plant. Whether *A. petiolata* creates a similar zone of inhibition for black morels in the soil is unknown, and depends on multiple factors including whether *A. petiolata* continuously deposits enough inhibitory allelochemicals and whether black morels are inhibited in the soil environment. *Alliaria petiolata* leachates were only introduced once in this experiment, so the effect of continuous or multiple leachate additions was not tested. Continuous or multiple leachate introductions could prevent cultures from overcoming the zone of inhibition. If roots continuously secrete allelochemicals, then *A. petiolata* could potentially suppress black morel growth in the soil.

Whole plant and shoot leachates varied in the responses by species and by day. For the shoot leachate, yellow morels developed the line trait in greater numbers over time with the most cultures expressing this trait on day five. Shoot leachate also elicited the pigment trait in yellow morels, but this trait did not appear in the cultures for the first few days, and developed over time (Figure 11). The whole plant leachate did not elicit the line trait in yellow morels but did elicit the pigment trait in a significant number of cultures on day four (Figure 10). After day four, the number of

yellow morel cultures expressing this trait dropped. It seemed that the entire culture was producing more pigment over time and the mycelium surrounding the disc produced pigment a little sooner than the rest. But the rest of the culture produced similar amounts of pigment and the area around was similarly pigmented. Whole plant and shoots leachates did not elicit the pigment and line traits for black morels.

One surprising result was that the isothiocyanate treatment did not have any discernible effect on either the yellow cultures or the black cultures. We presumed the ITC treatment would be a positive control because previous studies by Cantor *et al.* (2011) and Wolfe *et al.* (2008) showed suppression of three ectomycorrhizal fungi at these concentrations (AITC 0.001 mM and BITC 7.5 nmol/ml). Our result could have been due to improper preparation and mixing of the treatment. It is possible that BITC and AITC do affect morels at higher concentrations but no tests were conducted. Further experiments could address this question. There is a possibility that *A. petiolata* allelopathy toward morels is based on multiple chemicals working synergistically. Isothiocyanates are typically accompanied by their precursor glucosinolates, and *A. petiolata* is known to also produce isovitexin, alliarinoside, and hydrogen cyanide (Cipollini & Gruner, 2007; Haribal & Renwick, 1998, 2001). These additional compounds, or a synergistic interaction between them, could play a role in allelopathy. It is quite possible that these compounds elicited the responses in our experiment.

Our experiment used living *A. petiolata* rosettes collected in the fall, and the root leachate affected *Morchella* more than whole plant and shoot leachates. It is expected that allelochemicals would be produced in the roots because they have more

contact with the soil than shoots, but not all studies show this pattern. For example, Vaughn and Berhow's (1999) found that shoots and leaves had very low levels of glucosinolates in the fall and little to no glucosinolates in the spring. Root tissues consistently contained glucosinolates in both spring and fall, and they had higher levels of benzyl glucosinolate than shoots. Another study compared the effects of *A. petiolata* shoots and roots on germination of three North American woodland plants (Cipollini & Flint, 2009). Cipollini and Flint (2009) found that *A. petiolata* shoots suppressed germination more so than roots. Barto *et al.*'s results (2009) showed that shoots and roots produce different chemicals or varying concentrations of the same compounds, and indicated that allelochemical concentration in the soil is highest when *A. petiolata* plants are germinating and in high densities. Lankau (2009) showed that allelochemical concentration varies by population age.

Exhibition of Traits by Clade

The line trait was more likely to appear in black morel cultures than in yellow morel cultures. Yellow morel cultures given either root or shoot leachates expressed the line trait but only after a few days. Black morel cultures expressed the line trait when given any of the leachates. When given shoot leachate, the line trait disappeared in black cultures. Both yellow and black morels expressed this trait, but their proportions were different and the patterns of when the trait was expressed was very different between these two clades (Figure 11). Black morel cultures expressed the line trait in 0.33 of cultures on the first day and fell to 0.13 by day seven. Yellow morel cultures was exhibited by 0.41 cultures on the first day, but the number rose to

0.54 by day seven. It is likely that in nature black and yellow morels respond differently to *A. petiolata* over time.

The pigment trait appeared in both black and yellow morel cultures, but once again there were differences between the clades. The black cultures only expressed the pigment trait when given root leachate. The yellow cultures expressed the pigment trait when given either root or shoot leachate, but not the whole plant leachate. The yellow cultures given root leachate expressed the pigment trait all seven days in statistically significant proportions, and the number of yellow morel cultures given shoot leachate did not reach statistical significance until day six. Exhibition of the pigment trait increased in all three leachate treatments over time in both black and yellow morels.

Black morel cultures were more likely to form, and briefly maintain, a zone of inhibition compared to yellow morel cultures. Many cultures that formed a zone of inhibition eventually overcame the zone, and the trait disappeared over time (Figure 9). If in nature *A. petiolata* continually produces enough allelochemical to suppress black morel growth, then they could be affected. Further studies on both the release of allelochemicals and how black morels respond to *A. petiolata* in natural settings are needed.

The traits recorded in this experiment were chosen because they were easily identifiable changes that occurred in the cultures after leachate was added. The zone of inhibition trait is pertinent because it could indicate that black morels are inhibited by *A. petiolata* in nature. *Monascus sp.* increases pigment production when stressed (Babitha *et al.*, 2007). Whether increased pigment production is a sign of stress in

Morchella is possible but unconfirmed. The pigment seen in this experiment could be melanin, but no tests were done to confirm this. The line trait is the most poorly understood. The line itself seemed to be composed of a higher density of hyphae (Figure 14). It was very common for the line and the pigment trait to appear simultaneously in black cultures in the root extract (Figure 9). The mechanism behind this trait is unknown, as is what triggers the mechanism. Further studies are needed to understand why the line and pigment traits were expressed, and what they mean for *Morchella* growth and success.

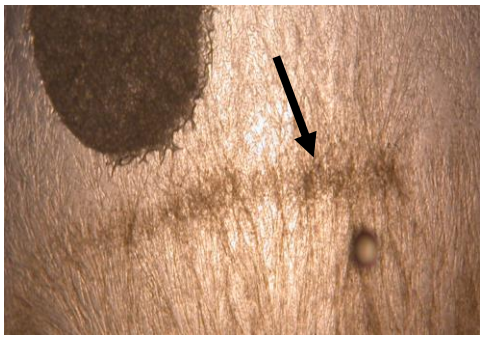

Figure 14. An example of the line and pigment traits. The arrow is point to a portion of dense mycelium with more pigment.

Contextualizing Results

Previous studies on *A. petiolata*'s effects on mycorrhizal fungi have generally found that *A. petiolata* suppresses fungal growth (Callaway *et al*., 2008; Cantor *et al.*, 2011; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). These studies documented how *A. petiolata* extracts—or soil conditioning—lower spore viability and root colonization (Callaway *et al.* 2008; Stinson *et al.*, 2006; Wolfe *et al.*, 2008). Most studies focused on arbuscular mycorrhizal fungi (AMF) and the plants reliant on them. Callaway *et al.* (2008) and Stinson *et al.* (2006) conditioned soils with *A. petiolata*, inoculated the soil with AMF, and planted native North American plants in the soils. The plants and

soils were studied for colonization, infectivity, and plant biomass; all were lowered with *A. petiolata*. Cantor *et al.* (2011) supported the two studies by showing that a low concentration of allyl isothiocyanate was associated with lower AMF spore germination. Barto *et al.* (2010) found that in one plant species, *A. petiolata* extracts led to lower seed germination and pre-symbiosis growth but did not affect symbiosis formation and post-symbiosis growth. Wolfe *et al.* (2008) is the only published study that reported on *A. petiolata*'s effects on ectomycorrhizal fungi (EMF). Their experiments showed that benzyl isothiocyanate suppressed spore germination, and that EMF colonization was lower when *A. petiolata* was growing nearby.

Our study's design differed greatly from these previous studies, looking at very different traits. The results suggest that *A. petiolata* could affect black morels. The zone of inhibition in black morel cultures suggests that *A. petiolata* could suppress vegetative growth. Future experiments are needed to see if the *in vitro* effects are also seen in more natural conditions, and more experiments are needed to understand how all *Morchella* life stages are affected.

Future Experiments

Greenhouse studies could show how *A. petiolata* affects *Morchella*'s mycorrhizal relationships. Harbin and Volk (1999) discovered *Morchella*'s mycorrhizal status by placing a *Morchella* culture in a Petri dish in which a small seedling was also growing. Harbin demonstrated the relationship was mycorrhizal by cross sectioning the roots, and by using DAPI dye to reveal nutrient exchange. Similar experiments could be replicated, but with an added *A. petiolata* treatment. Growing *A. petiolata* in the Petri environment with *Morchella* and a host species

could show whether the relationship is disrupted or if nutrient exchange is affected. This experimental design would allow us to compare yellow morels and black morels' growth and mycorrhizae formation when *A. petiolata* is introduced.

The effect of *A. petiolata* would best be illustrated by studying other life stages. A germination experiment would be best for understanding whether *Morchella* spread and early growth is affected. It would be useful to study sclerotia formation and growth emerging from a sclerotia to fruit. In TJV's anecdote, *A. petiolata* invaded a site where black morels were already established. Thus, *A. petiolata* interacted with vegetative hyphae, sclerotia, and the fruiting body. It is difficult to study if and how *A. petiolata* affects *Morchella* fruiting, because fruiting is difficult to predict and control.

On the molecular level, it would also be useful to discover what proteins and mechanisms are affected by *A. petiolata*. Thus, it would be possible to better measure and quantify the traits that are responsible for pigment, line, and zone of inhibition traits. These experiments could reveal why different *Morchella* species responded differently to *A. petiolata* leachate.

Field studies will be most useful to understand the relationship between morels and *A. petiolata*. Morel hunters could record whether they find morels under associated tree species where *A. petiolata* is present or absent, but it is difficult to rule out other factors affecting presence or absence. There are no described molecular primers for finding and identifying *Morchella* mycelium in the soil. Tools could be developed to test for *Morchella*'s presence or absence in soil or amongst

ectomycorrhizae. These tools could reveal whether *Morchella* forms mycorrhizae in nature, and if *A. petiolata* affects presence or absence.

Conclusion

Our experiment has shed some light on how *A. petiolata* affects *Morchella*. It helped lay groundwork for future experiments because we now know more about what kind of traits can and do appear. These results can provide a basis for studying *Morchella*'s response in different life stages, as well as responses in greenhouse and field studies. The zone of inhibition trait could be measured in soil environments by using nitrocellulose film as did Cantor *et al.* (2011) in their study. Measuring pigment could be useful in such experiments, but other experiments are needed to understand how this trait affects *Morchella* growth and survival.

The results of this study indicate that *A. petiolata*'s roots produce more allelochemicals than shoots. These results are in keeping with previously reported results. These findings could help inform future experiments on identifying which compounds that *A. petiolata* produces are allelopathic and studying how the allelochemicals are released in the soil. These results will potentially serve to lay some groundwork for future studies on *A. petiolata* allelopathy, and its effect on this economically and culturally important fungus.

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